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Polyphyly of non-bioluminescent *Vibrio fischeri* sharing a *lux*-locus deletion

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Summary

This study reports the first description and molecular characterization of naturally occurring, non-bioluminescent strains of *Vibrio fischeri*. These 'dark' *V. fischeri* strains remained non-bioluminescent even after treatment with both autoinducer and aldehyde, substrate additions that typically maximize light production in dim strains of luminous bacteria. Surprisingly, the entire *lux* locus (eight genes) was absent in over 97% of these dark *V. fischeri* strains. Although these strains were all collected from a Massachusetts (USA) estuary in 2007, phylogenetic reconstructions allowed us to reject the hypothesis that these newly described non-bioluminescent strains exhibit monophyly within the *V. fischeri* clade. These dark strains exhibited a competitive disadvantage against native bioluminescent strains when colonizing the light organ of the model *V. fischeri* host, the Hawaiian bobtail squid *Euprymna scolopes*. Significantly, we believe that the data collected in this study may suggest the first observation of a functional, parallel locus-deletion event among independent lineages of a non-pathogenic bacterial species.

Introduction

The *lux* locus – encoding all genes necessary and sufficient for bacterial bioluminescence – is found predominantly within the *Vibrionaceae*. Within this family of heterotrophic, aquatic gammaproteobacteria approximately 10 traditionally delineated species are known to include bioluminescent strains (Urbanczyk *et al*., 2008). In most members of the *Vibrionaceae*, it appears that light emission is the exception and not the rule; non-bioluminescent strains outnumber bioluminescent strains in all but a few *Vibrionaceae* species (Grim *et al*., 2008; Urbanczyk *et al*., 2008).

One *Vibrionaceae* species reported to be composed only of bioluminescent strains is *Vibrio fischeri*. Why, in all environmental samples analysed to date, have naturally occurring, non-bioluminescent (dark) *V. fischeri* never been reported? A reasonable hypothesis is that the trait of bioluminescence is consistently and strongly selected for in *V. fischeri* by their primary environmental habitat(s). One such *V. fischeri* habitat is their animal hosts (Farmer, 2006). *Vibrio fischeri* strains have been isolated in the greatest abundance from bobtail squids of the genera *Euprymna*, *Sepiola* and *Rondeletiola* (Cephalopoda: Sepiolidae), and fishes of the genera *Monocentris* and *Cleiodopus* (Actinopterygii: Beryciformes) (Ruby, 1977; Fitzgerald, 1978; Wei and Young, 1989; Nishiguchi *et al*., 1998). Indeed, bioluminescent *V. fischeri* were found to outcompete dark mutants during symbiosis initiation of juvenile bobtail squid (Bose *et al*., 2008), although this is the only study directly demonstrating a fitness benefit for bioluminescence in *V. fischeri*.

Best known for their mutualistic associations, *V. fischeri* have also been isolated as planktonic cells from both pelagic and neritic environments (see, for example, Baumann *et al*., 1971; Lee and Ruby, 1994). In contrast to host-associated environments, little is known about the ecology of these planktonic (or 'free-living') *V. fischeri* in marine sediment or seawater. Even less is known about *V. fischeri* bioluminescence (and selection for/against this trait) in these niches, although the density-dependent nature of bioluminescence induction via quorum sensing (Boettcher and Ruby, 1990) implies that *V. fischeri* populations must be above a certain density to produce light. In studies using either a 16S rRNA- (Jones *et al*., 2007) or a *luxA*-based *V. fischeri*-specific probe (Lee and Ruby, 1995), concentrations of this species in Hawaiian seawater and sediment have been estimated to be as high as 10^2–10^4 cells ml^{-1} (Boettcher and Ruby, 1990). Assuming even distribution of *V. fischeri*, these concentrations imply that bioluminescence autoinduction does not take place when cells are free-living in these environments, potentially mitigating any selection for or against this trait among planktonic *V. fischeri*.

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A second, contrasting hypothesis is that non-bioluminescent *V. fischeri* have not been previously sampled because these sampling efforts were biased in favour of the isolation of bioluminescent strains. Originally, *V. fischeri* was grouped under the genus *Photobacterium* by Beijerinck when he isolated bioluminescent bacterial colonies from the Dutch coast, reflecting the idea that *V. fischeri* were bioluminescent bacteria *ipso facto* (Beijerinck, 1889). More exacting taxonomic efforts by Baumann and associates, consisting of a more cosmopolitan collection of *V. fischeri* strains, also reported bioluminescence to be a hallmark of *V. fischeri* (Reichelt and Baumann, 1973). Finally, more contemporary *V. fischeri* isolation efforts have either sampled from host-associated environments exclusively (Ruby and Nealson, 1976; Jones *et al*., 2006; Urbanczyk *et al*., 2007; Wollenberg and Ruby, 2009) or used *lux* gene-specific probes to identify and isolate planktonic *V. fischeri* (Lee and Ruby, 1992).

Here we describe *V. fischeri* strains collected in 2007 from several different microhabitats within an estuary environment at Ipswitch, MA. The work below probes the molecular nature of bioluminescence loss among these strains, describes the phylogenetic relationship of these dark strains to light-producing *V. fischeri*, and assesses the relative fitness of these strains in the *Euprymna scolopes* light organ.

Results

The majority of V. fischeri *isolated from PIE (Plum Island Estuary) do not produce bioluminescence, in contrast to all previously studied* V. fischeri *strains*

During 2007, over 1750 *Vibrionaceae* isolates were collected from various sources (e.g. water-column particles and invertebrate tissues) in an estuary north of Boston, Massachusetts – PIE (Plum Island Estuary). Three conserved loci (malate dehydrogenase – *mdh*; adenylate kinase – *adk*; heat-shock chaperonin – *groL*) were partially sequenced from each of these strains (Preheim *et al*., 2011). These sequences, plus their homologues from four additional, well-characterized *Vibrionaceae* (*V. fischeri* ES114 and MJ11; *V. logei* SA6; *V. salmonicida* LFI1238) were concatenated and used to construct a neighbour net (Fig. 1).

From this reconstruction, a clade of approximately 70 strains was found to include *V. fischeri* ES114 and MJ11. Strains from this clade were independently confirmed to be *V. fischeri* using the hvnC-PCR assay (Wollenberg and Ruby, 2009), which demonstrated a positive pair of products for all strains tested in this putative *V. fischeri* group, but a negative (single) product both for closely related strains not found in this group and for other, more distantly related *Vibrionaceae* (Figs 1 and 2A; data not shown). Furthermore, partial 16S *rrn* sequencing and BLAST searching/alignment of several of these putative *V. fischeri* strains resulted in nearest matches to sequences from known *V. fischeri* (data not shown). During manipulation of these 70+ PIE *V. fischeri* strains, colonies growing either on standard marine agar medium or in log-phase liquid cultures produced no visible bioluminescence (Table 1), in contrast to the majority of *V. fischeri* studied to date.

One hypothesis explaining non-visible bioluminescence in the 2007 PIE *V. fischeri* is that these strains underproduce autoinducer. The majority of well-studied and non-visibly bioluminescent *V. fischeri* strains are known to secrete little of the autoinducer 3-oxo-hexanoyl homoserine lactone (3-oxo-C6 HSL) under standard *in vitro* growth conditions (Nealson, 1977). Addition of 3-oxo-C6 HSL to liquid cultures increases bioluminescence in non-visibly bioluminescent *V. fischeri* several orders of magnitude (Boettcher and Ruby, 1990). However, 97% of the PIE *V. fischeri* were found to be unresponsive to autoinducer addition in liquid culture (Table 1; data not shown). Three strains, 9ZD146, 9CSC6, 9CSC146 were visibly bioluminescent in liquid and solid marine media, and did respond to autoinducer addition. Two of these strains, 9CSC6 and 9CSC146, were identical in all three partial loci sequenced above, making it possible that they are sibling isolates. Taken together, these results indicate that luminescence is a rare trait among *V. fischeri* populations in PIE.

Another hypothesis for the absence of bioluminescence by almost all PIE *V. fischeri* strains is the possible underproduction of the aldehyde substrate for the luciferase enzyme. Some strains of both *V. fischeri* and *V. salmonicida* have been reported to be non-visibly bioluminescent in liquid culture under normal growth conditions, but can produce visible light after the external addition of an aldehyde substrate like decanal (Fidopiastis *et al*., 1999; Lupp *et al*., 2003). Dark PIE *V. fischeri* strains did not produce any bioluminescence upon addition of exogenous decanal (Table 1; data not shown), suggesting that they are not limited by this substrate.

Non-bioluminescent PIE V. fischeri *share an identical, full* lux*-region deletion*

Another hypothesis explaining non-visible bioluminescence in PIE *V. fischeri* is the loss or mutation of some component of the *lux* region. The *V. fischeri lux* region consists of two divergent operons containing the eight structural and regulatory genes (Fig. S1) necessary for light production (Gray and Greenberg, 1992; Meighen, 1994). Surprisingly, all PIE *V. fischeri* strains that did not respond to autoinducer (with the exception of strain 9ZD146) also lacked PCR-amplification products from

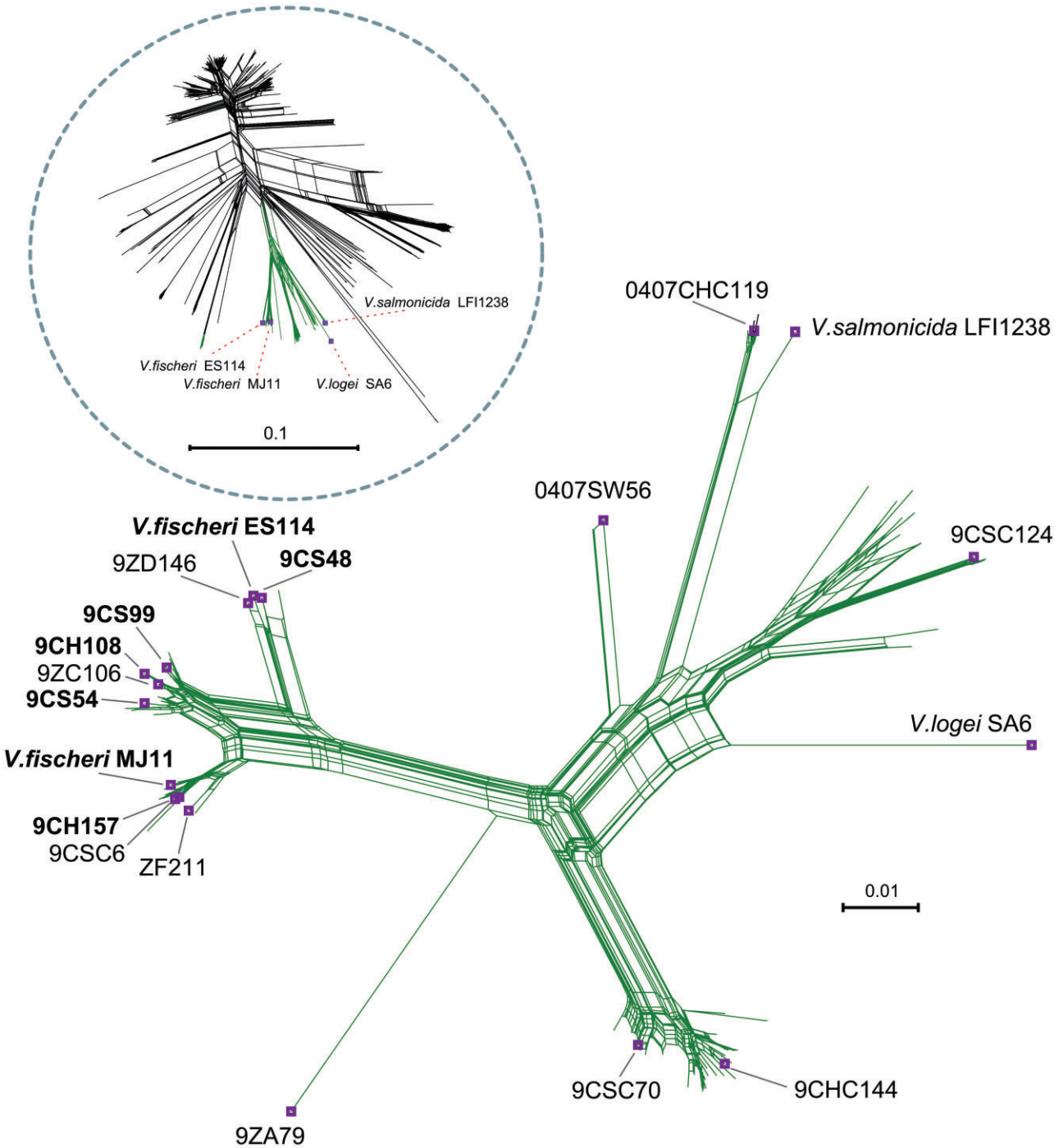


Fig. 1. Phylogenetic relationships among *Vibrionaceae* sampled from Plum Island Sound (PIE), Massachusetts in spring and fall of 2007. Both reconstructions depicted were created via the neighbour net method using uncorrected P distance, ordinary least-squared variance and splits calculated by the equal-angle algorithm. (Inset): Unrooted neighbour net constructed with concatenated, partial sequences from three loci (*mdh*, *adk*, *groL*) of over 750 *Vibrionaceae*, two *V. fischeri* (ES114 and MJ11), one *V. salmonicida* (LFI1238) and one *V. logei* (SA6) strain. Known *V. fischeri* strains were found among taxa on a defined branch (green). (Main figure): Unrooted neighbour net, constructed as above, from all taxa on the green branches in the inset. Fifteen isolates frequently referred to in the text and strains ES114, MJ11, LFI1238 and SA6 are marked with purple squares; *V. fischeri* taxa included in Fig. 3 are listed in bold. Bars represent substitutions per site, as listed.

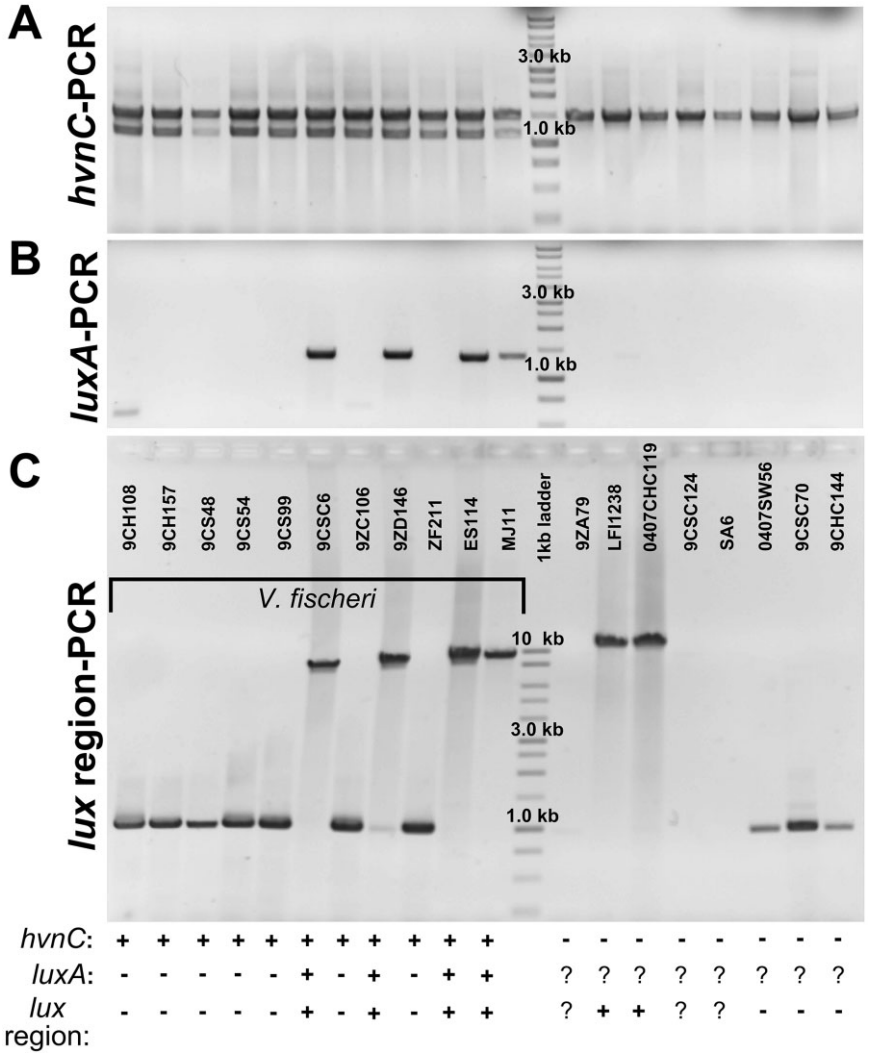


Fig. 2. Agarose gel electrophoresis of PCR products from *V. fischeri*- and *lux*-specific amplification assays demonstrates variability in *lux*-region amplification from PIE *V. fischeri* strains. For each strain, amplification of (A) *hvnC* (~1 kb) and a portion of the 16S *rrn* operon(s) (~1.5 kb), (B) *luxA* (~1.5 kb) and (C) the full-length *lux* region (~10 kb) and conserved housekeeping genes flanking a full deletion of the *lux* region (1 kb) was judged to be successful (+), unsuccessful (–) or inconclusive (?). (A and B) PCR reaction products were run on the same gel; this gel was cropped to remove wells. The same DNA stock was used for PCR reactions in the three gels pictured and Fig. S2.

both the gene encoding the alpha chain of bacterial luciferase (*luxA*) and the entire *lux* region (Fig. 2; data not shown).

Genomic sequences from a subsample of PIE *V. fischeri* revealed that all contained a full deletion of the *lux* region between intact sequences of the two well-conserved flanking loci (i.e. *ribG*- and *mscS*-like genes) (data not shown). Unassembled contigs covering the full genome of one of these strains (ZF_211 – S. Timberlake, M.F. Polz and E.J. Alm, pers. comm.) were assembled using *V. fischeri* ES114 and MJ11 full-genome sequences as scaffolds by the progressive Mauve algorithm in Mauve (Darling *et al*., 2010). This assembly and exhaustive BLASTing of *V. fischeri* ES114 *luxA* nucleotide sequence against these contigs indicated no evidence of a *lux* region anywhere in the ZF_211 genome assembly (Fig. S1, data not shown).

Analysis of a related locus was used to identify the extent of disruption of the general autoinduction/quorum-sensing pathway in dark PIE *V. fischeri*. The octanoyl-L-homoserine lactone (C8-HSL) receptor and synthase locus (i.e. the *ainRS* operon) could be amplified from all dark PIE *V. fischeri* (Fig. S2; data not shown). Thus, while the downstream portion of the *V. fischeri* quorum-signalling pathway (the *luxRICABDEG* genes) appears to be missing in dark PIE *V. fischeri*, the upstream, non-*lux*, portion (e.g. *ainRS*) that is shared with other *Vibrionaceae* (Lupp *et al*., 2003) is present. All of these molecular

Table 1. Phenotypic and genotypic bioluminescence properties of *V. fischeri* strains from Plum Island Estuary collections.

	Isolate	*hvnC* PCR	*lux* operon PCR	*luxA* PCR	*ain* operon PCR	RLU[a] SWT	RLU[a] SWT + C6	RLU[a] SWT + decanal
V. fischeri	*V. fischeri* ES114	+	+	+	+	38	$> 10^4$	2582
	V. fischeri MJ11	+	+	+	+	$> 10^4$	$> 10^4$	$> 10^4$
	ZF211	+	–	–	+	3	1	1
	9ZC106	+	–	–	+	2	1	4
	9ZD146	+	+	+	+	3	5	4
	9CSC6[b]	+	+	+	+	$> 10^4$	$> 10^4$	$> 10^4$
	9CS48	+	–	–	+	2	1	1
	9CS54	+	–	–	+	3	2	2
	9CS99	+	–	–	+	2	1	3
	9CH157	+	–	–	+	2	0	1
	9CH108	+	–	–	+	2	1	1
	9CSC70	–	–	?[c]	+	1	2	2
	9CHC144	–	–	?	+	1	1	1
	0407SW56	–	–	?	?	1	1	2
	V. logei SA6	–	?	?	+	$> 10^4$	$> 10^4$	$> 10^4$
	V. salmonicida LFI1238	–	+	?	+	34	41	$> 10^4$
	0407CHC119	–	+	?	+	2	7	$> 10^4$
	9CSC124	–	?	?	+	$> 10^4$	$> 10^4$	$> 10^4$
	9ZA79	–	?	?	?	1	1	2

a. Relative luminescence units (RLU) measured in response to growth in SWT medium, SWT + 120 nM 3-oxo-C6-HSL autoinducer (C6) and SWT + 0.01% aldehyde (decanal). Values of $> 10^3$ are above the upper limit of detection for the luminometer; values of approximately 5 or less are the lower limit of detection/background for the luminometer for the given sensitivity setting. Similar values were recorded in three independent experiments; representative values from a single experiment are presented.
b. The bioluminescence properties of isolate 9CSC146 were found to be identical to those of isolate 9CSC6 for all assays listed.
c. Either cryptic (or no) amplification products observed for given PCR temperature settings and primers.

results are summarized for a representative subsample of *V. fischeri* strains in Table 1.

Non-bioluminescent PIE V. fischeri *are polyphyletic within a phylogenetic reconstruction of the* V. fischeri *species complex*

One hypothesis explaining the conserved *lux*-region deletion that is characteristic of dark PIE *V. fischeri* is that these strains are all closely related, and share a most recent common ancestor within the *V. fischeri* species lacking the *lux* operon; in other words, non-bioluminescent PIE *V. fischeri* are monophyletic. To test this hypothesis, both dark PIE *V. fischeri* ($n = 5$) and bioluminescent *V. fischeri* from our laboratory collection ($n = 11$) were sequenced at four conserved housekeeping loci (recombinaseA – *recA*; malate dehydrogenase – *mdh*; catalase – *katA*; dihydroorotase – *pyrC*). Maximum parsimony, maximum likelihood and Bayesian methods were used to construct, and determine the statistical significance of, evolutionary relationships among these *V. fischeri* strains. In all reconstructions, non-bioluminescent PIE *V. fischeri* strains were distributed throughout the phylogeny and were not monophyletic within either of the two *V. fischeri* subclades (Figs 3 and S3). The hypothesis that non-bioluminescent *V. fischeri* form a monophyletic clade was statistically rejected via parametric bootstrapping

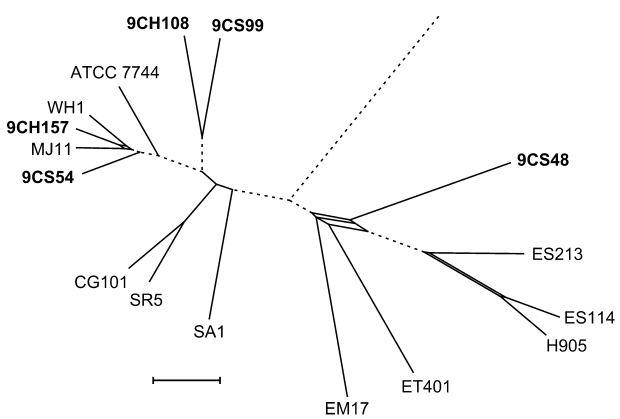


Fig. 3. Non-bioluminescent and *lux*-region absent taxa are polyphyletic within a phylogenetic reconstruction of *V. fischeri*. Consensus network inferred from 10 000 phylograms produced by ClonalFrame analyses of 16 *V. fischeri* taxa using a concatenation of four loci: *recA*, *mdh*, *katA* and *pyrC*. ClonalFrame was used to infer genealogical relationships from posterior distributions of 2000 samples from five independent runs. This aggregate ClonalFrame posterior distribution was used by SplitsTree4 to build a consensus network. For this network, reticulate relationships found in at least 20% of the samples are depicted as parallel branches in parallelograms; nodes supported by a posterior probability of greater than 95% (as summarized in a consensus tree by ClonalFrame) are at the termini of stippled branches. The root (stippled, unlabelled branch) has been truncated in order to expand the main *V. fischeri* group and contains both outgroup taxa: *V. salmonicida* LFI1238 and *V. logei* SA6. Bold isolates lack evidence for the *lux* operon and are phenotypically non-bioluminescent. The bar represents 0.01 substitutions per site.

($P < 0.01$). Furthermore, from the original neighbour net of all PIE *V. fischeri* (Fig. 1), the five listed bioluminescent *V. fischeri* strains (i.e. ES114, MJ11, 9CSC6, 9CSC106 and 9ZD146) appeared interspersed among the broader *V. fischeri* group. All of these results strongly support the hypothesis that non-bioluminescent *V. fischeri* do not share an exclusive most recent common ancestor within the *V. fischeri* species.

STCBS-based isolation from seawater does not significantly bias estimation of bioluminescent V. fischeri *abundance relative to LBS-based isolation*

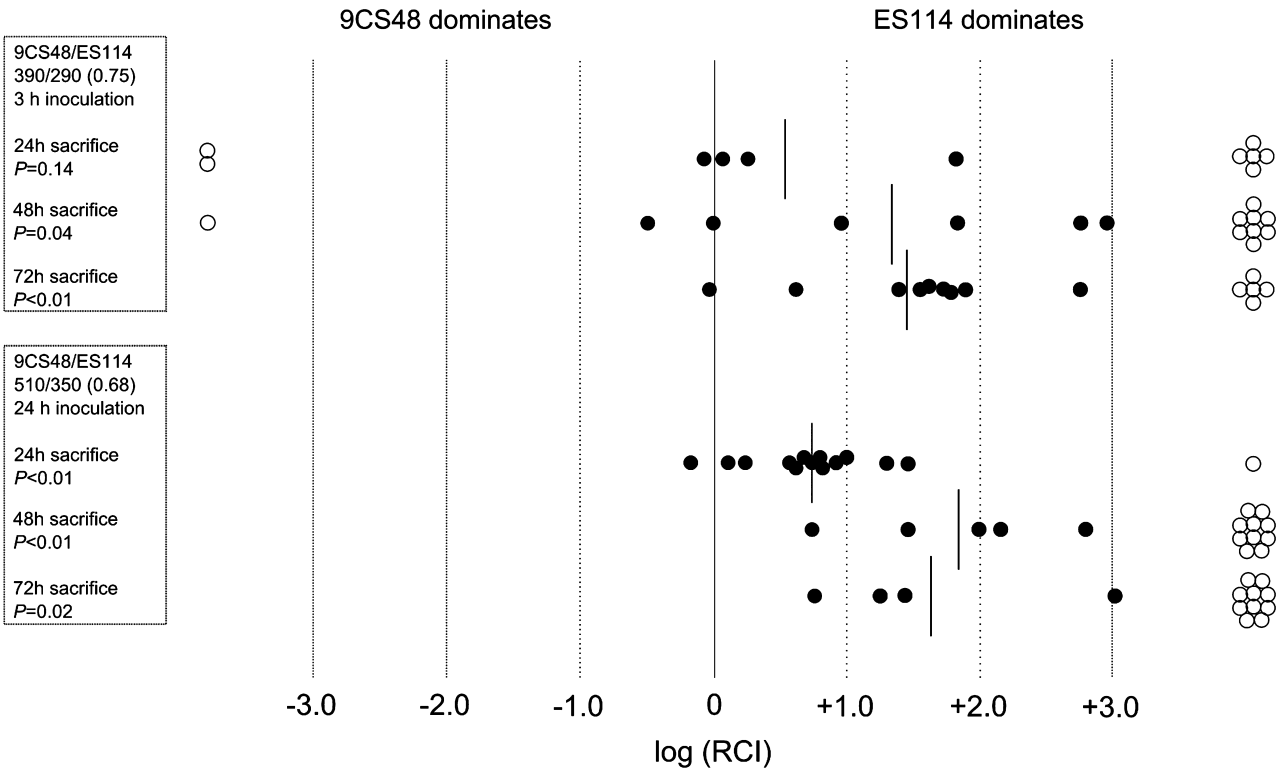
An interesting observation from the above results is the high abundance of non-bioluminescent, *lux* operon-deleted, *V. fischeri* strains in the PIE sampling relative to both bioluminescent strains in this same sampling and all other worldwide samplings. Because sampling regimes over the past century had previously failed to isolate non-bioluminescent *V. fischeri*, and it has been noted that TCBS medium selects against some species of *Vibrionaceae* (Farmer, 2006), we asked whether the isolation procedure using STCBS agar medium was responsible for the high abundance of non-bioluminescent *V. fischeri* in the 2007 PIE study. A randomly chosen subsample of both bioluminescent and non-bioluminescent *V. fischeri* strains was cultured in sterile-filtered Instant Ocean for 24 h before being spread on either LBS agar or STCBS agar media (Fig. S4). While mean culturable bioluminescent and non-bioluminescent *V. fischeri* concentrations were significantly smaller on STCBS agar relative to LBS agar ($F_{1,78} = 70.8502$; $P < 0.001$), for both media there was no significant difference between the mean cfu ml^{-1} of either bioluminescent or non-bioluminescent strains ($F_{1,18} = 0.3729$; $P = 0.55$). Similarly, there was no significant interaction detected between growth medium and a strain's bioluminescence state ($F_{1,78} = 0.3219$; $P = 0.57$). These results strongly suggest that, although the use of STCBS decreases the total number of *V. fischeri* recovered from marine samples, this medium is not biased in its recovery of cells of either bioluminescent or non-bioluminescent *V. fischeri*.

Non-bioluminescent PIE V. fischeri *are outcompeted by native, bioluminescent* V. fischeri *in the Hawaiian bobtail squid model*

Several studies have analysed the role that the *lux* operon plays in the mutualistic relationship between *V. fischeri* and the Hawaiian bobtail squid, *E. scolopes* (Visick *et al*., 2000; Bose *et al*., 2008). These studies have detailed the relative failure of a genetically engineered mutant (with either one or all *lux* structural genes deleted) to compete or persist in symbiosis with juvenile hosts. We asked whether two naturally occurring, dark PIE *V. fischeri* (strains 9CS48 and ZF211) also have a defect during colonization of the *E. scolopes* light organ. When competed against either of the dark strains for 1–3 days (Fig. 4), the native, bioluminescent symbiont strain ES114 became dominant over the non-bioluminescent strain between 48 and 72 h; these results demonstrate a marked competitive advantage of the native symbiont over the dark PIE strain in symbiosis accommodation and persistence. Another native, bioluminescent symbiont, strain ES213, showed a similar competitive advantage over both strains 9CS48 and ZF211 after 48 and 72 h (data not shown). Finally, the *lux* region-containing, C6- and decanal-unresponsive PIE strain, 9ZD146, was also outcompeted by both ES114 and ES213 during colonization (data not shown).

One explanation for the observed competition defects of the dark PIE strains might be a general inability to survive in the juvenile squid after 48 h; for example, it is known that the competition defect of an ES114 mutant missing the structural genes of the *lux* operon is related to its inability to persist at high abundance in the light organ at 48 h (Bose *et al*., 2008). This hypothesis was supported by an evaluation of the mean abundance of each of the four strains during single-strain colonization over 72 h (Fig. S5). Like genetically engineered dark mutants, at 48 and 72 h, both strains ZF211 and 9CS48 exhibited a qualitatively lower mean abundance per squid when compared with the native symbionts ES114 and ES213. Nevertheless, we recognize there may be other factors, in addition to their lack of luminescence, that could contribute to a competition defect in the PIE strains.

Fig. 4. Non-bioluminescent *V. fischeri* isolates from the PIE collections are outcompeted by the bioluminescent strain ES114 during colonization of *E. scolopes* juveniles. Each box represents a single experiment and contains nine descriptive lines: the first line contains strain names, which are above the initial cfu ml^{-1} of each isolate with the initial ratio of ES114 to the competitor in parentheses; the third line details the number of hours aposymbiotic *E. scolopes* were exposed to the *V. fischeri* competition mixture. The fourth through ninth lines detail the time of sacrifice of a given cohort of juveniles (total time post hatching) and the *P*-value (*P*) for a one-sample Student's *t*-test statistic given the null hypothesis that mean logarithm of the relative competition index [log(RCI)] is zero. To the right of each of these lines are a series of closed and open circles, each closed circle represents the log(RCI) calculated from the homogenate of a single juvenile squid. Open circles represent a squid's light-organ homogenate containing only a single strain (the other strain's concentration was at least 1000-fold lower than the recovered strain's concentration). The mean log(RCI) value for each experiment (excluding open circles) is represented by a vertical bar.


9CS48 dominates
ES114 dominates
9CS48/ES114
390/290 (0.75)
3 h inoculation
24h sacrifice
P=0.14
48h sacrifice
P=0.04
72h sacrifice
P<0.01
9CS48/ES114
510/350 (0.68)
24 h inoculation
24h sacrifice
P<0.01
48h sacrifice
P<0.01
72h sacrifice
P=0.02
-3.0
-2.0
-1.0
0
+1.0
+2.0
+3.0
log (RCI)

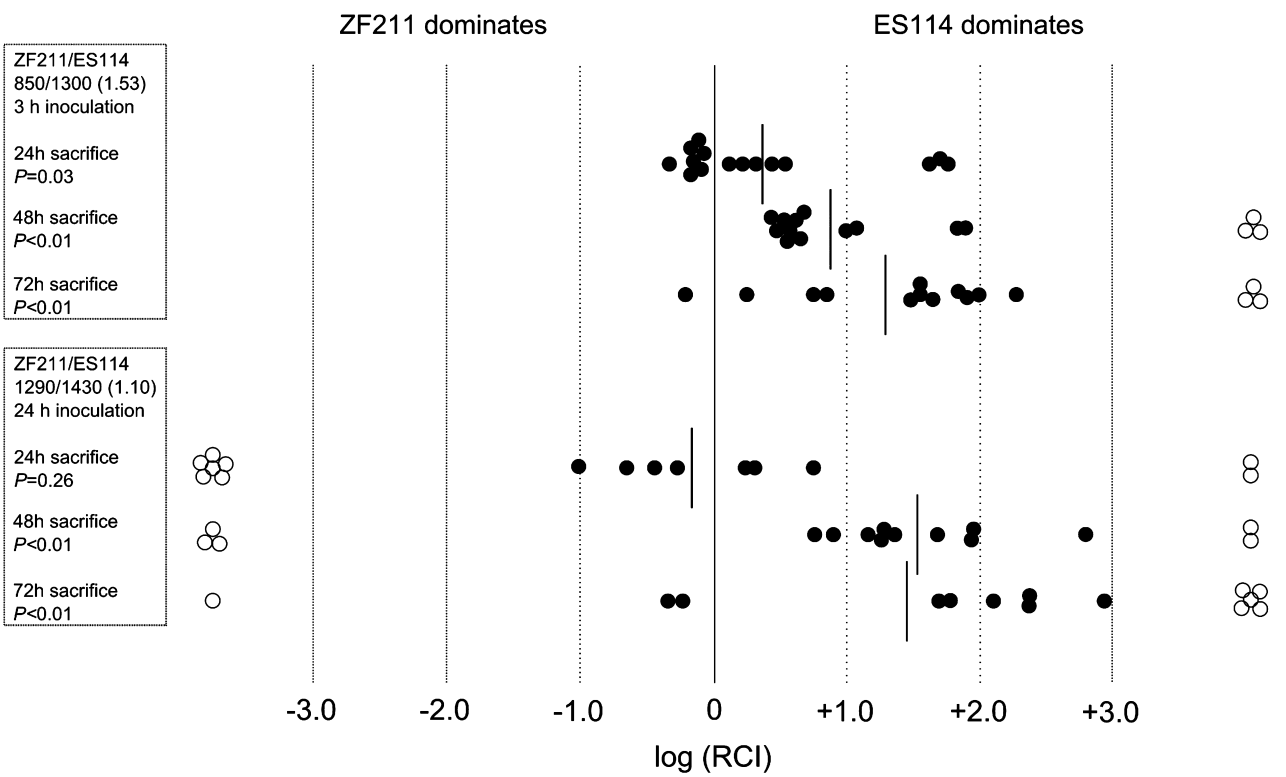

ZF211 dominates
ES114 dominates
ZF211/ES114
850/1300 (1.53)
3 h inoculation
24h sacrifice
P=0.03
48h sacrifice
P<0.01
72h sacrifice
P<0.01
ZF211/ES114
1290/1430 (1.10)
24 h inoculation
24h sacrifice
P=0.26
48h sacrifice
P<0.01
72h sacrifice
P<0.01
-3.0
-2.0
-1.0
0
+1.0
+2.0
+3.0
log (RCI)

Table 2. Evolutionary scenarios for *lux*-region homoplasy among *V. fischeri*.

		Evolutionary event	
		Natural selection	Genetic drift
Event direction	Gain	Natural selection for *lux*-region gain	Gain of *lux* region through genetic drift
	Loss	Natural selection for *lux*-region loss	Loss of *lux* region through genetic drift

Discussion

This study is the first description of non-bioluminescent *V. fischeri*. The work suggests that broader, relatively unbiased environmental samplings may increase the general knowledge of the bioluminescence spectrum for any given species of *Vibrionaceae*. Samplings of both *Vibrio cholerae* and *V. harveyi* support this conclusion; such studies have demonstrated that non-bioluminescent isolates of luminous species are as abundant, or more abundant, than bioluminescent isolates in marine environments (Grim *et al*., 2008; O'Grady and Wimpee, 2008; Zo *et al*., 2009).

From the intraspecific phylogenetic reconstruction, we conclude that the presence of a region of functional *lux* genes (i.e. the genomic trait underlying bioluminescence) is a homoplasy within the species *V. fischeri* (Fig. 3). This conclusion implies that, rather than being a single event in the evolutionary history of this species, gain or loss of the *lux* region appears to have occurred multiple times in multiple *V. fischeri* subgroups in an identical manner. To recapitulate the observed pattern of luminescence and *lux*-region distribution among extant strains of *V. fischeri*, a 2×2 matrix of four general evolutionary scenarios might be hypothesized (Table 2).

We favour the hypothesis that selection has led to identical loss of the eight *lux* genes among unrelated, *V. fischeri* strains sampled from the PIE environment. In this scenario, *lux*-region presence is ancestral to *V. fischeri* and we would predict there to be evidence of selection in this region for extant *V. fischeri* strains. Most strongly, data from a previous report (Bose *et al*., 2008), as well as this study (Fig. 4), demonstrate that the *lux* genes provide a fitness advantage in the light organ of the Hawaiian bobtail squid, *E. scolopes*. Additionally, patterns of nucleotide substitution among the *luxA* sequences of different *V. fischeri* strains, and comparison of fully sequenced *lux* regions between a fish-associated and a squid-associated strain, suggest that selection has been operating on this bioluminescence enzyme-encoding locus (Mandel *et al*., 2009; Wollenberg and Ruby, 2011). Finally, recent studies of experimental evolution in non-native *V. fischeri* colonizing juvenile *E. scolopes* have demonstrated that the light organ can strongly, and rapidly, select for particular bioluminescence levels (Schuster *et al*., 2010).

We are unaware of a parsimonious model resulting from genetic drift that would produce an identical loss/gain of the *lux* region necessary to recapitulate these observed data. For example, if genetic drift were combined with *lux*-region loss, we might expect to have observed strains of *V. fischeri* that, like non-bioluminescent *V. harveyi* isolated from seawater, contained point mutations or small indels in *lux* genes that effectively abrogate light production (O'Grady and Wimpee, 2008). In this study, only a single strain (9ZD146) was found to possess a non-functional, but intact, *lux* region (Table 1; data not shown). The observation of this one possible *lux* pseudogene in PIE *V. fischeri* might support the hypothesis of drift followed by a strong deletional bias of the *lux* locus.

Another, alternative hypothesis is that recombination has affected the evolutionary history of these isolates. First, recombination among the housekeeping loci used in this analysis may have obfuscated 'true' evolutionary relationships among *V. fischeri* strains. Scrambling of these strains' evolutionary history could generate the homoplasy observed for the *lux* region. We believe this hypothesis is not the most parsimonious explanation of these data for the following two reasons. First, others have demonstrated, with different housekeeping loci (Ast *et al*., 2009), intraspecific phylogenetic reconstructions of *V. fischeri* showing two major clades. Our results (Figs 1 and 3), recapitulate this same two-clade pattern, with similar strains in each clade; this suggests that the general phylogenetic signal observed among these three, independent reconstructions is fundamental to *V. fischeri*. Second, we have used these housekeeping loci in other studies (Mandel *et al*., 2009; Wollenberg and Ruby, 2011) in which phylogenetic reconstructions of *V. fischeri* were predictive of physiological differences among extant strains in both seawater and host-associated environments. If recombination had truly obscured the phylogenetic signal in these two studies, it is unlikely that the phylogenetic and experimental/biological data would be complementary.

Another hypothesis is that homologous recombination of the (deleted or intact) *lux* locus has occurred among disparate *V. fischeri* lineages. Phylogenetic reconstructions of these isolates using their *lux* loci will be instructive for assessing the relative similarity or difference between housekeeping- and bioluminescence-operon evolution in

this lineage. Results of such a study might be able to confirm or refute the hypothesis that this operon has 'moved' among *V. fischeri* lineages. It will be more difficult to assess the recombination of deleted sequence among *V. fischeri*, although the analysis of conserved, flanking sequences might prove useful.

Among the possible scenarios for *lux*-region gain or loss in response to natural selection, the existing data most strongly indicate loss. We propose that adaptive radiations of non-bioluminescent *V. fischeri* have been facilitated by selection for the loss of light production and deletion of the *lux* region. In contrast, multiple events of identical *lux*-region gain appear unlikely. Molecular data suggest that *luxA* (Wollenberg and Ruby, 2011) and *lux*-region (Urbanczyk *et al*., 2008) sequences from *V. fischeri* are more similar to each other than to those of other *lux* genes found in the *Vibrionaceae*. These data are most consistent with a common ancestry for the *lux* region in *V. fischeri*, and weaken the argument of multiple, distinct gains of this locus as the species has diverged through time: this latter scenario provides no explanation for why and how integration of the *lux* region into exactly the same position in the chromosomes of multiple lineages of *V. fischeri* might occur.

Adaptive radiation resulting in the loss of an entire gene has been observed among bacteria, and has been best documented from genomic analyses of pathogens (Lawrence, 2005). Large and/or complete deletions have been found in temporally disparate radiations of bacteria grown in diverse environments ranging from laboratory media to *Homo sapiens* (Nakata *et al*., 1993; Maurelli, 2007; Barrick *et al*., 2009; Zdziarski *et al*., 2010). Interestingly, however, reports of identical locus-deletion events among phylogenetically distinct lineages of a single bacterial species are sparse.

One such observation describes three or four deletions found among different lineages of *Mycobacterium tuberculosis* (Tsolaki *et al*., 2004). The authors argue that there may be selection for several of these deletions: the missing regions encode drug sensitivity and 'latency genes' that are hypothesized to impede disease progression and transmission. In other words, loss of these genes during adaptive radiation(s) provides a fitness advantage to unrelated *M. tuberculosis* strains within the host in response to selection.

Our data provide the first evidence for an identical functional-locus deletion among different lineages of a non-pathogenic bacterial species. Such conserved *lux*-region deletions among unrelated *V. fischeri* could imply that selection for the loss of this locus (and, thus, bioluminescence) provides a fitness advantage outside of the squid or fish light-organ environment. Data from a previous study suggests that isogenic mutants lacking the *lux* operon's structural genes experience a fitness advantage relative to brightly bioluminescent *V. fischeri* during growth in rich medium (Bose *et al*., 2008) due to the significant metabolic cost of light production (Karl and Nealson, 1980). Other studies have indicated that bioluminescent *V. fischeri* also exhibit survival differences in natural seawater (Wollenberg and Ruby, 2011), although similar experiments have not yet been performed with non-bioluminescent *V. fischeri*.

One hypothesis consistent with the results described here is that one (or more) PIE habitats (Preheim *et al*., 2011) (e.g. *Hemigrapsus sanguineus* and *Mytilus edulis* guts, or zooplankton-, plant- or seawater-associated niches) exerts selection against the presence of the *lux* region and/or bioluminescence in *V. fischeri* cells. However, a previous analysis of *Vibrionaceae* spp. partitioning among habitats sampled in the 2007 PIE study found no correlation between any defined niche and the increased abundance/presence of *V. fischeri* (Preheim *et al*., 2011). Therefore, there exists no evidence that *V. fischeri* strains lacking the *lux* region are endemic to, or particularly abundant in, any specific PIE habitat. Additional non-biased sampling in both PIE and other marine environments will help to distinguish between chronic and ephemeral low-abundance populations of *V. fischeri* with *lux*-region heterogeneity.

If these PIE environments are not the source(s) of some or all non-bioluminescent *V. fischeri* carrying the *lux*-region deletion, what is? We hypothesize that the gut of an as yet unsampled marine animal is the most likely environment for the following four reasons. First, *V. fischeri* strains are known to be present in relatively high abundance the guts of many species of marine animals, where luminescence has no known function (Nealson and Hastings, 1979; Ruby and Morin, 1979). Second, abundant genomic and metabolic evidence suggests that most members of the *Vibrionaceae*, including *V. fischeri*, may be well adapted to the generally anaerobic, chitin-provisioned gut tract of a marine animals (Ruby *et al*., 2005; Hunt *et al*., 2008). Third, the abundance of *V. fischeri* in a nutrient-rich environment, such as a marine animal's gut, may lead to autoinduction of light production; as others have noted (Bose *et al*., 2008; Urbanczyk *et al*., 2008), the phenotype/genotype of light production can only be selected against if it is occurring. Finally, the microbial community present in any marine animal's gut might provide interspecific competition for light-producing *V. fischeri*, potentiating selection for more energetically economical, non-bioluminescent strains.

In conclusion, we find that the most parsimonious and well-supported hypothesis explaining luminescence and *lux*-region homoplasy among *V. fischeri* to be one in which PIE *V. fischeri* strains have evolved by natural selection for *lux*-region deletion. Although environmental sampling using STCBS does not appear to enrich for non-

bioluminescent *V. fischeri* strains (Fig. S4), the combination of this approach with *V. fischeri*-specific probes may improve their isolation (Lee and Ruby, 1992) and culture-independent enumeration (Jones *et al*., 2007). Additional single-locus and whole-genome data will be useful for following patterns of gene gain or loss among *V. fischeri* isolates; these data will also be invaluable for testing predictions made by more sophisticated conceptual frameworks. For example, a recently proposed source-sink model relates single-gene haplotype network structures to hypotheses of ecological relationships among closely related bacterial strains (Sokurenko *et al*., 2006); this technique might be useful in the identification of ecological reservoirs of dark *V. fischeri* in future sampling efforts. Finally, the continued use of the squid *E. scolopes*, as well as the development of better experimental seawater and marine-animal gut environmental models, will aid in the accurate measurement of fitness costs and benefits accruing to different *V. fischeri* strains. Applications of these approaches will enlighten both the study of the evolution and ecology of microbial bioluminescence and the process of intraspecific diversification among closely related bacteria.

Experimental procedures

Bacterial collection

A collection of *Vibrionaceae* isolated from various sources in Plum Island Sound Estuary, Ipswich, MA (PIE) in 2007 (Preheim *et al*., 2011) was the primary source of the majority of isolates used in this study. All initial 2007 isolations were made on STCBS: thiosulfate-citrate-bile salts-sucrose agar medium (Difco) amended with 10 g l^{-1} NaCl, for a total of 20 g l^{-1} of NaCl. Primary STCBS isolates were subcultured several times and stored in glycerol stocks at –80°C in a laboratory collection. Eleven *V. fischeri* strains collected worldwide over the last two decades, as well as nine potential *V. fischeri* and six closely related strains from the above sampling, and two closely related outgroups (*V. logei* SA6 and *V. salmonicida* LFI1238) were used for many of the comparative analyses described below (Table S1).

Bioluminescence assays

Single-strain bacterial cultures were grown at 28°C in seawater-tryptone (SWT) medium (Boettcher and Ruby, 1990) to stationary phase (optical density at 600 nm greater than 1.0; approximately 8 h) and measured for light production using a TD20/20 photometer (Turner Designs, Sunnyvale, CA) by recording relative luminescence units. Relative luminescence here means that each measured value is used for relative comparisons among strains, but that the measured value itself has arbitrary units. Next, these cultures were split and either (i) diluted into SWT containing 120 nM synthetic 3-oxo-C6-HSL (3-oxo hexanoyl homoserine lactone – Sigma-Aldrich Corp., St. Louis, MO), grown via shaking at 28°C to early logarithmic phase (optical density at 600 nm ≈ 0.1–0.3; approximately 1–2 h), and assayed for light production as above, or (ii) mixed with the luminescence substrate decanal (Sigma-Aldrich Corp., St. Louis, MO) at a final concentration of 0.01%, and immediately assayed for light production as above.

16S hvnC *PCR*

16S *hvnC* PCR was used as described previously (Wollenberg and Ruby, 2009) to identify *V. fischeri* strains by the differential separation of (i) two amplified products (*V. fischeri*: a ~1 kb *hvnC* and a ~1.5 kb 16S products) or (ii) one amplified product (other bacteria: only a ~1.5 kb 16S product) using standard agarose gel electrophoresis.

The originally reported set of PCR primers was found to be insufficient to amplify *hvnC* out of all putative *V. fischeri* strains from the 2007 PIE sampling (data not shown). Upon study of the newly sequenced *V. fischeri* MJ11 genome (Mandel *et al*., 2009), the *hvnC* reverse primer was found to contain a sequence that would be predicted to mismatch the targeted *hvnC* sequence of MJ11. A new *hvnC* reverse primer (hvnC1rvA 5′-CCAACAATAAGAGCTGAACG-3′) was designed and, in subsequent tests (data not shown), found to successfully and specifically amplify the desired portion of the *hvnC* locus from all *V. fischeri* available to us in a manner similar to that previously detailed (Wollenberg and Ruby, 2009).

PCR of the lux *region*

lux *region.* For each 12.5 μl of PCR reaction, the PCR mixture contained the following components: 8.75 μl of water, 1.25 μl of 10× Platinum HIFI *Taq* Buffer (Invitrogen, Carlsbad, CA), 0.5 μl of 50 mM $MgSO_4$, 0.25 μl of 10 mM dNTP mix (Promega, Madison, WI); 0.063 μl of each of two primers (below); 0.05 μl (0.25 U) of Platinum *Taq* HIFI DNA Polymerase (Invitrogen); 1.25 μl (5 ng) of DNA. Amplification was performed with the following programme: 95°C for 2 min; 30 repetitions of 94°C for 30 s, 55°C for 30 s, and 68°C for 10 min; 68°C for 10 min. Primers mscS5′_fw (5′-GTGAAGARTTT ATTGARGTAGC-3′) and matE5′_rv (5′-TGGATGAATCT GCATGAAATG-3′) target two conserved, 'anchor' loci for the amplification of a ~10 kb segment containing the entire *lux* operon. Sequencing of the (deleted) *lux* region was performed on 2007 PIE strains as described above, using the mscS5′_fw and matE5′_rv primers listed above.

luxA. For each 12.5 μl of PCR reaction, the PCR mixture contained the following components: 8.0 μl of water, 2.5 μl of GoTaq Buffer (Promega, Madison, WI), 0.25 μl of 10 mM dNTP mix (Promega, Madison, WI); 0.063 μl of each of two primers (below); 0.05 μl (0.25 U) of GoTaq DNA Polymerase (Promega); 1.25 μl (5 ng) of DNA. Amplification was performed with the following programme: 95°C for 3 min; 30 repetitions of 94°C for 30 s, 52°C for 30 s, and 70°C for 1 min; 70°C for 10 min. Primers luxA_whole_fw (5′-ACAAGTAY WACWGTTAARGAGCG-3′) and luxA_whole_rv (5′-AAGTGR TGTTCAYWWACAAARGCAG-3′) were designed to amplify an approximately 900 bp product from the *luxA* locus in *V. fis-*

cheri. For all amplifications, primer stock concentrations were 150 pmol μl^{-1} and amplifications were performed using a PTC-200 cycler (MJ Research, Waltham, MA)

Phylogenetic analyses

The whole population data set (1753 isolates) from a previously described sampling effort (Preheim *et al*., 2011), comprised of sequence fragments of three housekeeping loci common to all *Vibrionaceae* – malate dehydrogenase (*mdh*: 422 nucleotides; GQ992288–GQ994040), adenylate kinase (*adk*: 405 nucleotides; GQ990535–GQ992287) and the heat shock protein/chaperonin (*groL*: 427 nucleotides; GQ988782–GQ990534) – were concatenated as *mdh_adk_groL* to create a 1254 bp fragment, and combined with similarly concatenated sequences from two sequenced strains of *V. fischeri* (ES114 and MJ11), an additional unpublished putative *V. fischeri* strain from Plum Island Sound (ZF211), one *V. salmonicida* strain (LFI1238) and one *V. logei* strain (SA6).

This 1758-isolate data set was analysed to remove redundant sequences. Uncorrected P distances were calculated for all members of the remaining, non-redundant, 784-isolate data set; an unrooted split network was constructed from these distances using the neighbour net method with splits calculated using the equal angle algorithm, as implemented by SplitsTree 4 (http://www.splitstree.org). Split networks are phylogenetic reconstructions that do not assume a strictly tree-like relationship among extant taxa. Instead, ambiguity and uncertainty regarding relationships among taxa are represented by parallel edges (or 'splits') rather than single branches as in traditional 'tree-like' phylogenetic reconstructions (Huson and Bryant, 2006).

Five strains from the 2007 PIE collection found in the clade containing known *V. fischeri* strains (in the neighbour net reconstruction above; Fig. 1) were sequenced at four housekeeping loci as part of a previously described *V. fischeri* multilocus sequence typing scheme (Wollenberg and Ruby, 2011). All amplification and sequencing, proofing, trimming, concatenation and multiple sequence alignment of these loci was done as previously described in the above study. All new sequences have been stored in GenBank under Accession No. JF691567–JF691586 (Table S1).

The four loci were concatenated as *recA_mdh_katA_pyrC* to create a 2880-nucleotide-long fragment for each of the 18 strains listed in Table S1. For this collection of concatenated fragments, phylogenetic reconstructions were created using four methods: maximum parsimony, maximum likelihood, Bayesian inference and Clonal Frame reconstruction. The former three methods assume a tree-like structure of evolutionary patterning and are detailed in *Supporting information*.

Clonal Frame reconstruction, unlike the other three methods, both infers recombination events and reconstructs clonal relationships among taxa using Bayesian inference and the MCMC algorithm (Didelot and Falush, 2007). For the above data set, five independent runs of Clonal Frame 1.1 were performed; in each run, the 50% majority-rule consensus genealogy was estimated from the posterior probability distribution of 200 000 generations (thinning interval of 100) following burn-in of 100 000 generations. MCMC convergence was judged to be satisfactory by using the Gelman–Rubin test (Gelman and Rubin, 1992). The resulting posterior distribution of 10 000 samples (five runs of 2000 samples each) from each concatenated data set was analysed using a consensus network (Holland *et al*., 2004) with mean edge weights at a threshold of 0.2 and an equal-angle splits transformation as implemented in SplitsTree4 (Huson and Bryant, 2006).

Euprymna scolopes *colonization experiments*

Competition experiments were performed during colonization of juvenile *E. scolopes* using methods similar to those previously described (Wollenberg and Ruby, 2009). These experiments were performed by adding groups of 45 newly hatched, aposymbiotic hosts to individual bowls containing mixtures of two *V. fischeri* strains at a total concentration of approximately 10^3 cfu ml^{-1}. Seawater aliquots from each bowl were removed immediately before host addition, diluted and spread on LBS agar; resulting colony counts were averaged as an initial bacterial concentration for each strain. Host squid were left in bowls containing symbionts for either 3 h or 24 h. For 3 h experiments, squid were moved into individual scintillation vials, filled with 3 ml of sterile-filtered aquarium seawater (SFTW) after 3 h; for 24 h experiments, squid were immediately moved into individual scintillation vials filled with 3 ml of bacteria-inoculated bowl water. At 24, 48 and 72 h post hatching, the bioluminescence of 15 individual squid from each competition experiment was determined in a luminometer, and the animals anaesthetized with ethanol, blotted to remove seawater and frozen at −80°C for surface sterilization and sacrifice. The remaining squid were transferred to clean scintillation vials filled with 3 ml of SFTW. After 1–3 days at −80°C, frozen squid were removed into SFTW and homogenized; this homogenate was diluted and spread onto LBS agar for colony enumeration. *Vibrio fischeri* strains used in these competition experiments form colonies of either a white or a yellow colour on LBS agar (data not shown); the RCI (relative competition index) was calculated as previously described (Wollenberg and Ruby, 2011), using the ratio of abundance of one colour colony to the other. For each experimental time point, mean log(RCI) was calculated using all squid colonized by two strains; a one-sample Student's *t*-test was used to statistically assess the null hypothesis that mean log(RCI) is equal to zero. For the 12 experiments analysed in this way, this hypothesis was rejected at a level of $P < 0.05$ for all experiments at 48 h and 72 h post hatching.

Single-strain colonization experiments were performed as above with the following modifications: (i) groups of 30 aposymbiotic hosts were added to bowls containing one of four *V. fischeri* strains at a total concentration of approximately 10^3 cfu ml^{-1}, (ii) hosts were left in bowls containing symbionts for 24 h, and (iii) after removal from these bowls, hosts were incubated in 3 ml of SFTW for a total duration of between 24 and 72 h, with water changes every 24 h, as needed. Ten hosts per inoculated strain were sacrificed, homogenized, and plated on SWT agar at 24 h, 48 h and 72 h.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Full-genome comparisons among *V. fischeri* strains demonstrate that, in comparison with ES114 and MJ11, isolate ZF211 appears to lack the *lux* region, but contains other quorum-sensing loci. Multiple contigs from the ZF211 draft genome were aligned to the two existing *V. fischeri* genomes (i.e. MJ11 and ES114) using the progressive Mauve algorithm via Mauve 2.3.1 (Darling *et al*., 2010). Four genomic regions are depicted in each portion of the figure as follows: *lux* operon (A); *ain* operon (B); *luxO*, *luxU* and *qrr1* (C); *luxP* and *luxQ* (D). Comparisons are centred using a single ES114 locus (white vertical boxes and blue shading). Sequences shared by all three isolates are visualized by vertically coloured segments (i.e. red, green, orange or yellow); sequences (e.g. the *lux* operons) shared by two or fewer isolates are visualized by uncoloured gaps.

Fig. S2. All *V. fischeri* strains contain an amplifiable *ainRS* operon. Agarose gel electrophoresis of *ainRS* operon PCR amplification products. The major *ainRS* PCR product at ~4 kb is visible for all *V. fischeri* strains, and generally amplifiable from the non-*V. fischeri* strains and PIE isolates. Strain names (Table S1) are listed in each lane.

Fig. S3. Non-bioluminescent taxa lacking a *lux* region are polyphyletic within a phylogenetic reconstruction of *V. fischeri*. A 50% majority-rule consensus tree of 16 selected *V. fischeri* strains was constructed from a posterior distribution of approximately 5000 trees inferred by Bayesian methods using the GTR+I+Γ evolutionary model. The root leads to a clade containing the two outgroups: *V. logei* SA6 and *V. salmonicida* LFI1238 (not shown). The three numbers at different nodes (or identified by letter) represent clade-support values as calculated using the following methods: upper left, maximum likelihood bootstrap; upper right,

Bayesian posterior probability; lower centre, maximum parsimony bootstrap. Strains that lack the *lux* region and are phenotypically non-bioluminescent are indicated in bold. The bar indicates substitutions per site.

Fig. S4. Growth on TCBS agar is not significantly different between non-bioluminescent and bioluminescent *V. fischeri* strains. Bacteria were grown overnight in SFIO and spread on either LBS or STCBS agar plates. Colony counts were made from each medium for each strain. Bars represent mean colonies observed from 10 strains of either bioluminescent (white) or non-bioluminescent (grey) *V. fischeri*, with error bars representing 95% confidence intervals as described in materials and methods in *Supporting information*.

Fig. S5. Single-strain *V. fischeri* colonizations of juvenile squid demonstrate differences between the abundance of native symbionts (strains ES114 and ES213) and non-native, *lux* operon$^-$ strains (9CS49 and ZF211) at 48 and 72 h post inoculation. Each bar represents the mean cfu per squid of 10 squid inoculated with a single strain of *V. fischeri*, and sacrificed at 24, 48 or 72 h post inoculation; error bars represent 95% confidence intervals. Strain ES114: bright yellow bars; ES213: light yellow bars; 9CS48: dark grey bars; ZF211: light grey bars.

Table S1. *Vibrionaceae* strains used in this study.

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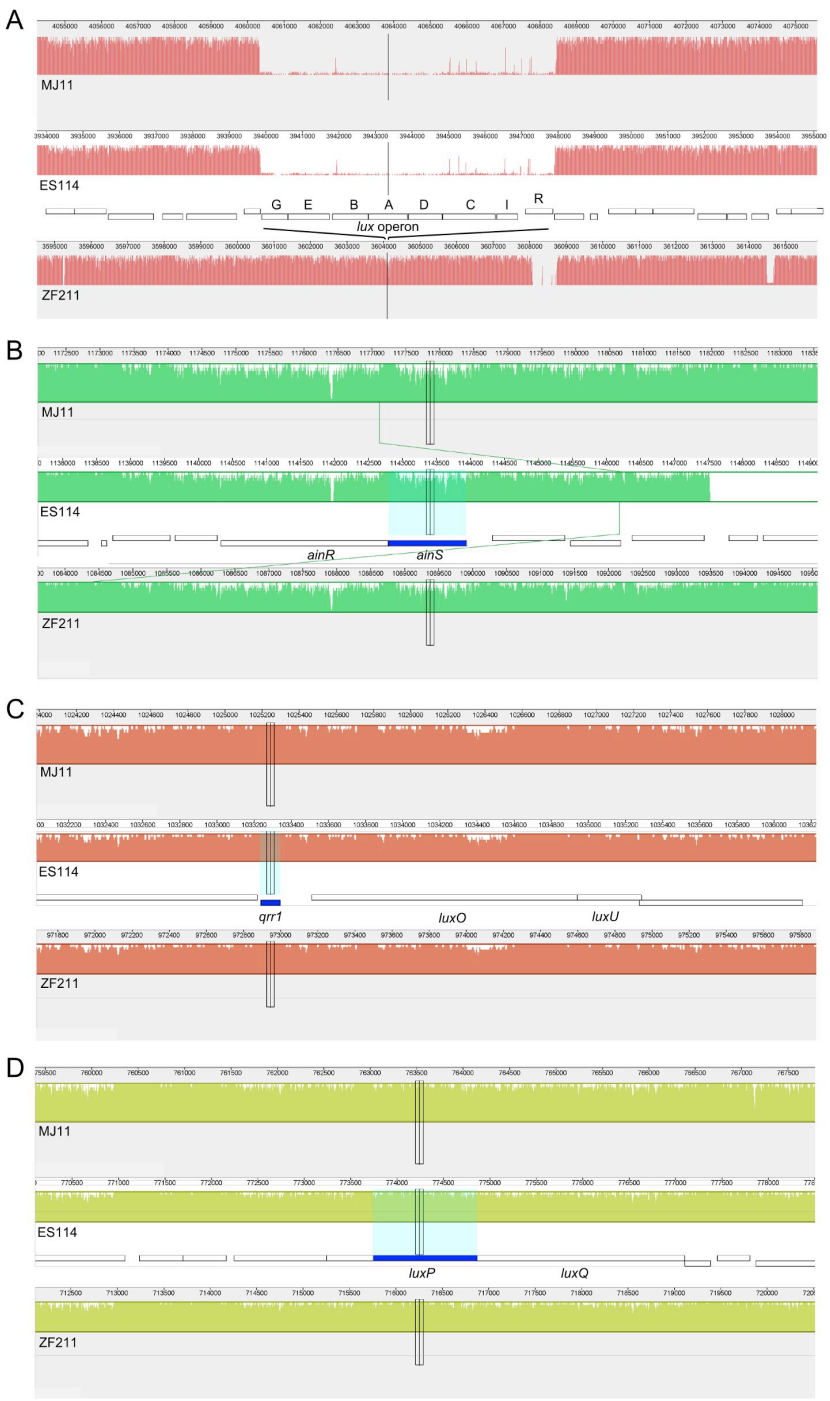
Supporting Figures and Tables

Polyphyly of Non-Bioluminescent *V. fischeri* Sharing a *lux*-Locus Deletion

M.S. Wollenberg, S.P. Preheim, M.F. Polz, and E.G. Ruby



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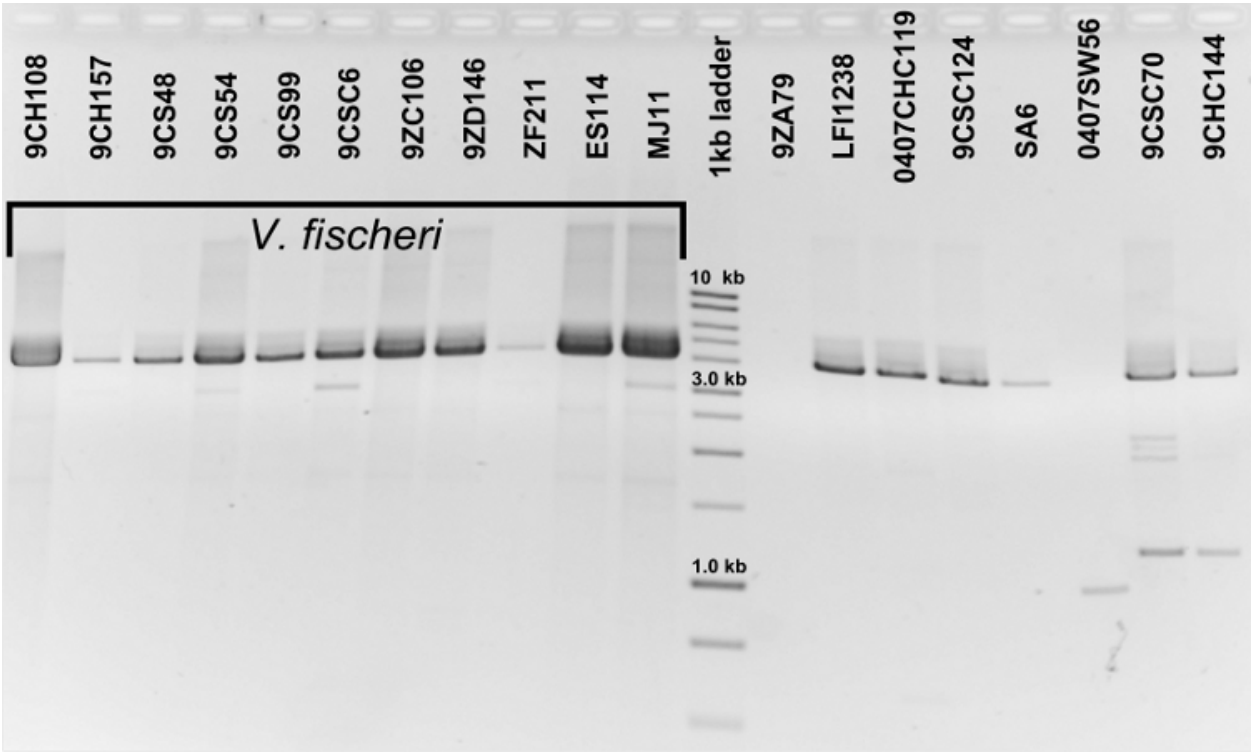
7



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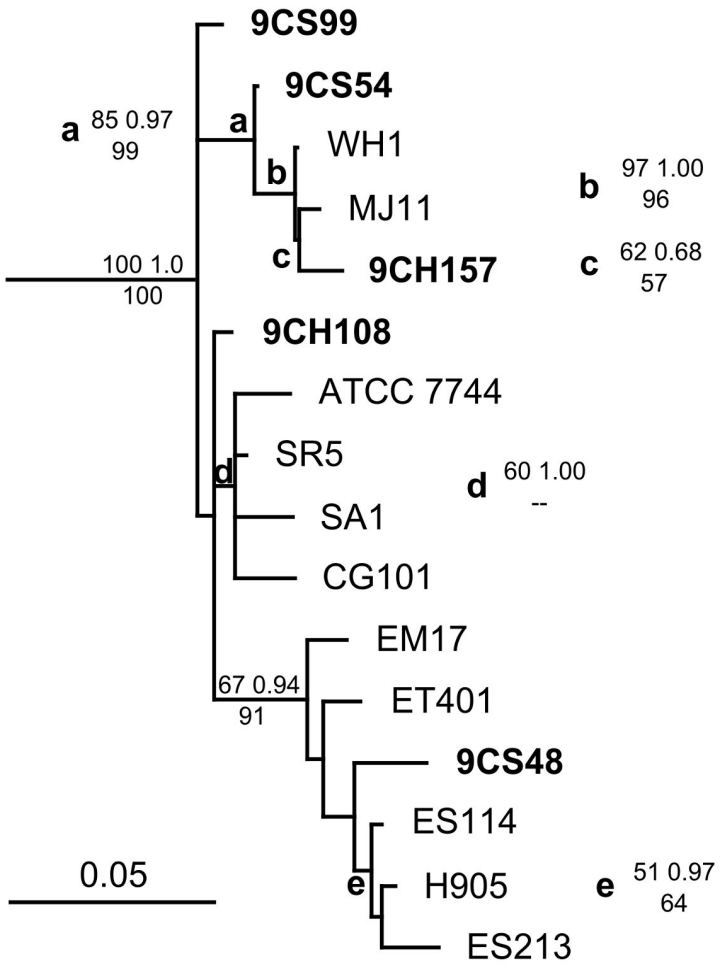
8



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9

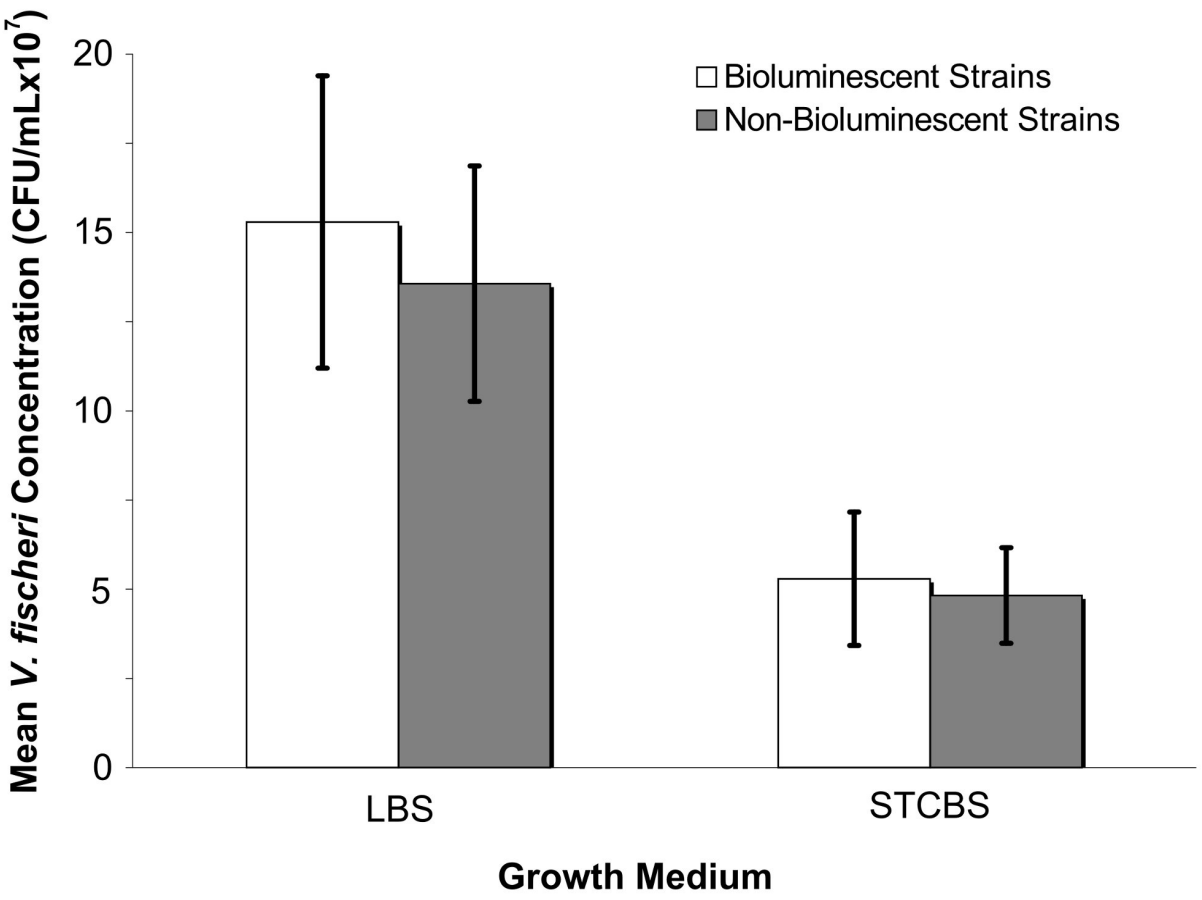

FIGURE S4. Growth on TCBS agar is not significantly different between non-bioluminescent and bioluminescent *V. fischeri* strains. Bacteria were grown overnight in SFIO and spread on either LBS or STCBS agar plates. Colony counts were made from each medium for each strain. Bars represent mean colonies observed from ten strains of either bioluminescent (white) or non-bioluminescent (grey) *V. fischeri,* with error bars representing 95% confidence intervals as described in the supporting information section's materials and methods.

10

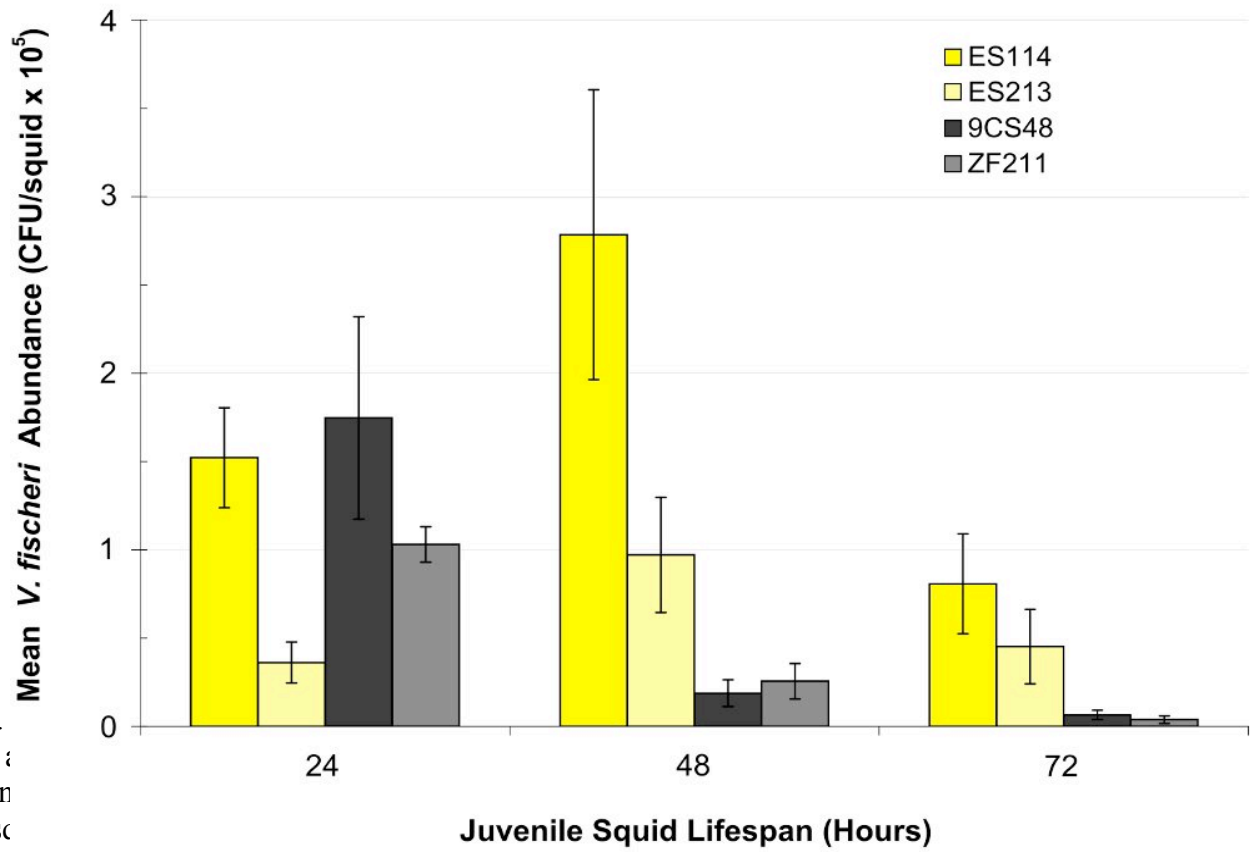

FIGURE S5. between the a operon strain mean CFU/sq 24-, 48- or 72-h post-inoculation; error bars represent 95% confidence intervals. Strain ES114: bright yellow bars; ES213: light yellow bars; 9CS48: dark grey bars; ZF211: light grey bars.

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